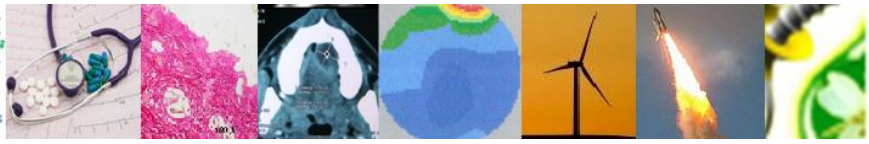

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RESEARCH: PLANT GENOMICS

IDENTIFICATION OF RAPD (RANDOM AMPLIFIED POLYMORPHIC DNA) MARKERS FOR ETHIOPIAN WILD *COFFEA ARABICA* L. GENETIC RESOURCES CONSERVED IN INDIA

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ABSTRACT

Ethiopian coffee (Coffea arabica L.) genetic resources conserved in India have not been subjected to molecular analysis. In the present study, fifteen wild coffee genetic resources representing eight populations and three cultivated varieties of Ethiopian origin were subjected to RAPD analysis. A total of 77 polymorphic RAPD bands were generated by seventeen random primers. The number of polymorphic bands detected with each primer ranged from 2 to 10 with a mean of 6.2 bands per primer. The amount of genetic variation among populations estimated by Shannon diversity indices were in the range of 4.02 to 4.3 indicating a moderate to relatively high level of diversity in the wild populations. Cluster analysis indicated geographical groupings for a few populations without any duplications among the germplasm studied. Present study adds new knowledge on Ethiopian Arabica coffee germplasm conserved in India that is of potential importance in developing a more comprehensive breeding programme for C. arabica and Gene Bank maintenance.

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Keywords: *Coffea; DNA markers; genetic diversity; germplasm; RAPD*

[I] INTRODUCTION

Coffee is one of the world's most valuable commodities, contributing largely to the economy of more than 50 countries of Asia, Latin America and Oceania. Of approximately 100 taxa of the genus Coffea (Family: Rubiaceae), only two species are economically important i.e., Coffea arabica L. and Coffea canephora Pierre ex Froehner, accounting respectively for about 70 percent and 30 percent of the world coffee production. In India the area under coffee cultivation is around 3,48,995 ha of which arabica and robusta account for 48 percent and 52 percent respectively[1].

Genetic consistency within varieties is essential for quality assurance of any agricultural product. Traditionally genetic diversity of genotypes was assessed based on differences in a range of expressions of morphological and agronomical characters. Currently, a variety of molecular techniques are available for measuring genetic diversity. The most common techniques are RAPD, RFLP, AFLP and SSR. All of them detect polymorphism by assaying subsets of the total amount of DNA sequence variation in a genome. However, they differ in principle, application, type and amount of polymorphism detected and cost and time requirements [2]. DNA polymorphisms are important in understanding the genetic variations in natural populations and can be used to explore issues of genetic diversity.

Cultivated varietal resources, utilized in coffee improvement programme posses a narrow genetic base. Thus, there is a need for widening of the existing genetic base by having more introductions especially from the centre of diversity [3]. Lack of variability may lead to vulnerability [4]. if, for example, new virulent pathogen races evolve. It is also important to understand that the adapted, elite germplasm does not contain all the most desirable alleles available in a

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species. Thus, utilization of the available germplasm in the gene banks assumes importance and their characterization is an urgent priority.

Development of coffee varieties of commercial importance in India followed the path of introduction, selection and hybridization. Exotic germplasm conserved in the Gene Bank should be characterized morphologically, biochemically and by molecular markers for further selection and integration of these wild progenitors in the main breeding program. Information about the genetic make-up of accessions helps decision making for conservation activities, which range from collecting and managing through identifying genes to adding value to genetic resources. The information from molecular markers or DNA sequences offers a good basis for better conservation approaches. In combination with morphological and biochemical characterization several DNA marker technologies have been developed and are available for genetic diversity studies [5]. Information on genetic diversity is also needed for the optimal design of plant breeding programmes, influencing the choice of genotypes to be crossed for the development of new plant varieties.

RAPD is the most commonly used marker system and is mainly used in studies assessing genetic diversity in natural plant populations. Adavantages of this technique include requirement of small amounts of template DNA; does not require sequence information for primer construction; is easy and quick to assay, has low cost, has random distribution throughout the genome and generates multiple fragments per reaction [6]. However, it is a dominant marker technique and has a problem of reproducibility of amplification [7]. and requires stringent optimization [8].

The specific objective of this study is to assess the genetic diversity among various accessions from different geographical locations of Ethiopia, maintained in CCRI Gene Bank so as to avoid maintenance of duplicate germplasm in the Gene Bank and possible utilization of the RAPD markers generated for future use in genetic improvement of arabica coffee.

[II] MATERIALS AND METHODS

2.1. Plant material

Fifteen accessions of coffee genetic resources with the entry numbers S.2440, S.2441, S.2600, S.2601, S.2602, S.2615, S.2604, S.2608, S.2642, S.2644, S.2649, S.2650, S.2707, S.2709 and S.2708 in the planting register of Botany Division, Central Coffee Research Institute (CCRI), growing in the Germplasm block of CCRI (Balehonnur, Chikmagalur District, Karnataka) were used as experimental material for RAPD studies. For DNA extraction young leaf tissue sample was used. The materials S.2440 and S.2441 were collected from Abyssinia during 1955 and the rest were from different geographical locations of Ethiopia collected in the wild during an F.A.O. sponsored expedition to Ethiopia in 1964 for exploration of coffee germplasm and the establishment of World Coffee Collections [9] **[Figure–1]**. Accessions S.2600 and S.2601 represent Harar province; S.2602 and S.2615 represent Shoa province; S.2604 and S.2608 represent Sidamo province; S.2642 and S.2644 represent Kaffa province; S.2649 and S.2650 represent Illubabor province; S.2707 and S.2709 represent Gojjam province and S.2708 represents Eritrea province **[Figure–1]**. The three cultivated types of Ethiopian arabica coffee *viz.,* Agaro, Cioccie and Tafarikela were included as comparative standards. At the time of leaf harvesting, plant materials of Abyssinian collections were 42 years old and rest of the collections including the comparative standards were 33 years old; all the genetic resources are healthy with good yield and vegetative growth.

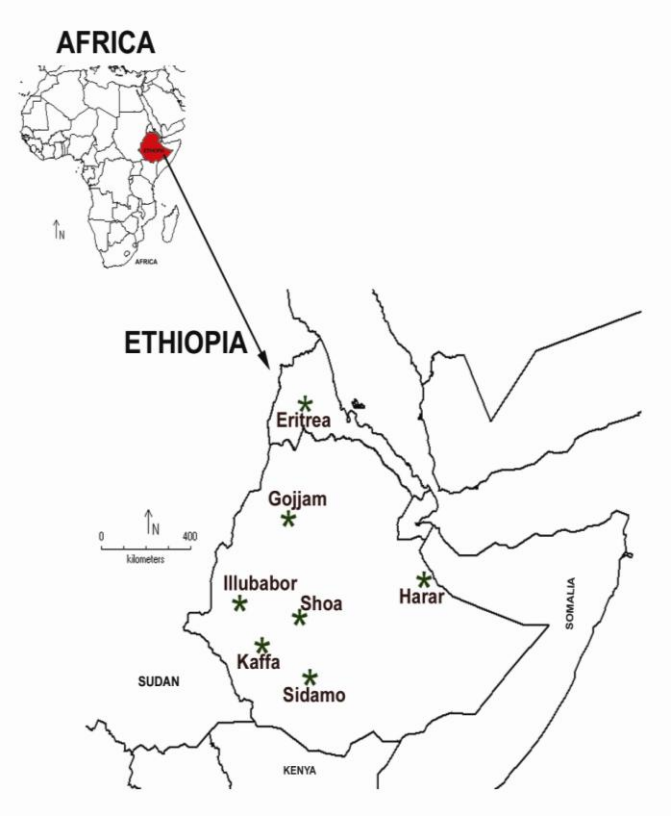

Fig: 1. Germplasm collection localities in Ethiopia during 1964

2.2. DNA extraction and purification

Total DNA was extracted from fresh young leaf tissue following the CTAB method with modifications suggested by Porebski et al. [10]. The protocol involves high salt precipitation, PVP, RNAse treatment followed by Phenol: Chloroform wash. Extracted DNA was purified by phenol extraction [11]. DNA quality was checked by electrophoresis in a 1 % agarose gel and the concentration was estimated in relation to the concentration of co-migration of □-phage DNA and by repeated measurements with spectrophotometer (Shimadzu, Japan) at 260nm.

2.3. Screening of RAPD primers

An initial screening of 140 oligonucleotide primers from Operon Technologies (CA, USA) was carried out, to identify primers that detect polymorphisms; only seventeen primers produced reproducible variation and were used in further analyses **[Table-1].**The reproducibility of the banding patterns of each primer was tested with respect to the template DNA and magnesium chloride concentration. Optimal conditions were determined for each specific primer, the conditions were strictly followed.

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Table: 1. Primers (Operon Technologies, Ca, USA) used for RAPD analysis and proportion of polymorphic bands, monomorphic bands, total number of bands and molecular size in different Ethiopian arabica germplasm

Primer	Primer sequence 5' to 3'	Total number of bands scored	Number of polymorphic bands	Number of monomorphic bands	Molecular size range (bp)
OPB07	GGTGACGCAG	5	4	1	800 - 1400
OPB09	TGGGGGACTC	6	3	3	300 - 1280
OPB12	CCTTGACGCA	2	1	1	820 - 1150
OPB18	CCACAGCAGT	3	3	0	740 - 1500
OPC02	GTGAGGCGTC	8	7	1	250 - 1700
OPE04	GTGACATGCC	4	6	1	240 - 800
OPE14	TGCGGCTGAG	7	3	3	870 - 1300
OPE15	ACGCACAACC	4	4	3	300 - 1800
OPE18	GGACTGCAGA	10	1	4	400 - 1400
OPF06	GGGAATTCGG	9	9	2	90 - 1300
OPF14	TGCTGCAGGT	10	7	1	500 - 1200
OPG08	TCACGTCCAC	2	1	1	800 - 1500
OPG11	TGCCCGTCGT	5	4	1	800 - 1400
OPG12	CAGCTCACGA	8	7	1	150 - 1700
OPG13	CTCTCCGCCA	6	3	3	430 - 1750
OPK05	TCTGTCGAGG	10	8	2	275 - 1250
OPP19	GTGGTCCGCA	8	6	2	250 - 1400
Total		107	77	30	--
Range		2-10	1 - 9	1-4	90 - 1800
Average		6.2	4.52	1.76	--

2.4. PCR amplification

The DNA amplification reactions were performed in a total volume of 25 µl containing 1x reaction buffer (75 mM Tris-HCl, pH 8.8, 20 mM (NH4)2SO4, 0.01 % (v/v) Tween 20), 1.5 mM MgCl2, 5 pmoles primer,

1.5 mM dNTPs (10 mM each of dATP, dCTP, dGTP and dTTP), 3U μl^{-1} of Taq polymerase (Bangalore Genei), and 25 to 30 ng of sample DNA. A master mix was prepared for each primer to minimize measurement deviation. The reaction mixtures were overlaid with two drops of light mineral oil.

Amplification was carried out in a Thermal cycler (Eppendorf, Germany) using programme profile consisting of an initial template denaturation at 95ºC for 5 minutes, followed by 42 cycles of one minute denaturation at 94ºC, one minute annealing at 36ºC and 2 minutes extension at 72ºC, with a final extension step of five minutes at 72ºC resulting in an exponential accumulation of specific fragments.

The PCR amplification products were separated on 1.4 % agarose gels containing 0.5 µg ml^{-1} ethidium bromide, and run in 1x TAE buffer (40 mM Tris acetate pH 8.0, 1 mM EDTA) at 90 volts.

2.5. Data scoring and analysis

Each amplified DNA fragment was considered as an independent character (locus), and scored as present (1) or absent (0). Those fragments that were monomorphic, not reproducible, appear in the control reaction or too difficult to score with certainty were excluded from the data analysis. Each amplified product was named by the code of the primer followed by its size in base pairs. Since RAPD markers are dominant, a locus was considered to be polymorphic if present in some and absent in other individuals of the population, and monomorphic if the bands were present in all individuals. No distinction was made between fragments of the same molecular size that varied in intensity. Estimation of genetic variation between the accessions was calculated from Shannon's diversity data. Shannon's diversity index is frequently used in RAPD data analysis because the index is insensitive to bias that may be introduced into data due to undetectable heterozygosity [12, 13]. The similarity matrices computed for each pair of populations were subjected to cluster analysis using STATISTICA for windows (Stat Soft Inc.1999, September 6).

[III] RESULTS

Of the 140 primers initially screened, RAPD patterns from seventeen primers were found to be reproducible and suitable for investigation **[Table–1, Figure–2]**. The seventeen oligonucleotide primers generated a total of 107 countable bands across 18 accessions representing 8 populations and three cultivated varieties. Of which 77 (71.9%) bands were stable (reproducible) polymorphic bands and 30 (28.1%) bands were monomorphic. The number of bands per primer varied from two (OPB12 and OPG08) to ten (OPE18, OPF14 and OPK05) with an average of 6.2 per primer, and the estimated molecular

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size was in the range of 90 to 1800 base pairs [Table–1]. Figure–2 depict the representative picture of the electrophoretic pattern of PCR amplified DNA fragments obtained during the analysis using these primers. The mean value for similarity indices represented by the eighteen coffee germplasm accessions ranged from 0.63 to 0.95 [Table–1] with an overall mean of 0.81, which means that the individuals from each coffee germplasm share, on average, 81 % of their RAPD fragments.

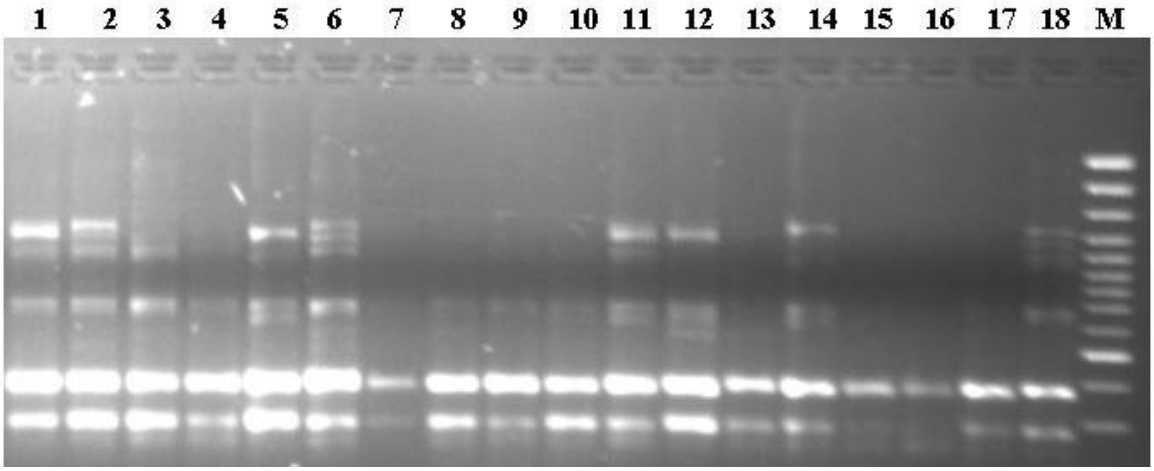

Fig: 2. Example of electrophoretic pattern of PCR amplified DNA fragments of wild coffee genetic resources produced by RAPD primer OPK5. (1- Agaro; 2-Cioccie; 3- Tafarikela; 4-S.2440; 5-S.2441; 6-S.2600; 7-S.2601; 8-S.2602; 9-S.2604; 10-S.2608; 11-S.2615; 12-S.2642; 13-S.2644; 14-S.2649; 15-S.2650; 16-S.2708; 17-S.2709; 18-S.2707; M-marker)

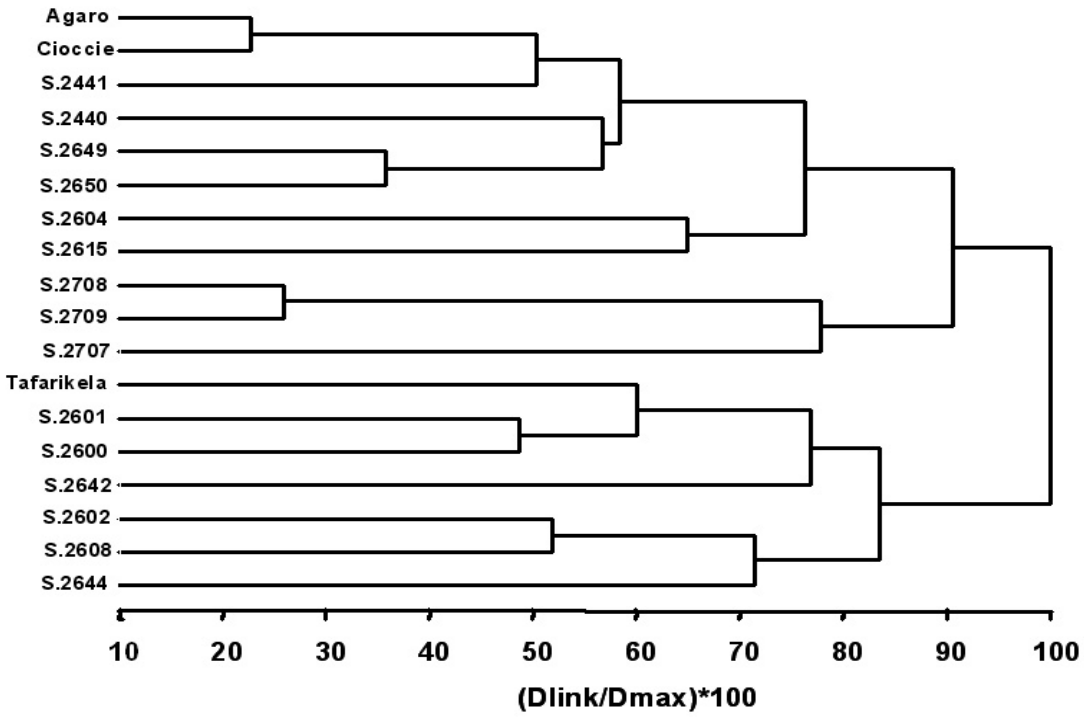

Fig: 3. Dendrogram generated by multivariate paired group Euclidean similarity measure by cluster analysis based on RAPD profiles in the Ethiopian germplasm

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Table: 2. Dice coefficient of similarity between the 18 germplasm from 5 different geographic locations of Ethiopia

	Agaro*	Cioccie *	Tafarikela *	S.2440	S.2441	S.2600	S.2601	S.2602	S.2604	S.2608	S.2615	S.2642	S.2644	S.2649	S.2650	S.2708	S.2709	S.2707
Agaro *	1																	
Cioccie *	0.95	1																
Tafarikela *	0.80	0.81	1															
S.2440	0.85	0.89	0.80	1														
S.2441	0.85	0.83	0.83	0.84	1													
S.2600	0.88	0.89	0.86	0.84	0.88	1												
S.2601	0.89	0.89	0.79	0.87	0.85	0.82	1											
S.2602	0.76	0.75	0.73	0.79	0.85	0.80	0.77	1										
S.2604	0.84	0.86	0.76	0.86	0.81	0.80	0.88	0.80	1									
S.2608	0.76	0.77	0.81	0.82	0.84	0.84	0.79	0.87	0.86	1								
S.2615	0.82	0.83	0.78	0.80	0.82	0.86	0.82	0.81	0.87	0.82	1							
S.2642	0.75	0.74	0.77	0.77	0.83	0.80	0.77	0.78	0.75	0.82	0.79	1						
S.2644	0.72	0.75	0.70	0.80	0.77	0.80	0.75	0.84	0.80	0.82	0.80	0.79	1					
S.2649	0.85	0.89	0.75	0.88	0.78	0.85	0.86	0.76	0.86	0.77	0.87	0.73	0.82	1				
S.2650	0.86	0.90	0.76	0.87	0.81	0.82	0.90	0.75	0.83	0.73	0.81	0.71	0.77	0.92	1			
S.2708	0.77	0.82	0.70	0.83	0.73	0.75	0.82	0.73	0.78	0.71	0.73	0.65	0.77	0.83	0.86	1		
S.2709	0.78	0.82	0.68	0.81	0.72	0.73	0.83	0.75	0.80	0.71	0.76	0.63	0.77	0.85	0.88	0.94	1	
S.2707	0.82	0.84	0.67	0.78	0.75	0.75	0.81	0.74	0.77	0.67	0.75	0.68	0.75	0.80	0.84	0.81	0.85	1

*Comparative standard

3.1. *Cluster analysis*

The result of the cluster analysis obtained using all the 18 germplasm produced two main clusters **[Figure–3]** of 11 and 7 accessions each. Cluster one consisted of Agaro, Cioccie, the material considered as standard for the study with S.2440, S.2441, S.2649, S.2650, S.2604, S.2615, S.2708, S.2709 and S.2707. Geographical groupings were noted for S.2440 and S.2441 from Abyssinia and S.2707 and S.2709 from Gojjam province in the sub-clusters of the first main cluster but not in pairs. Within the cluster one direct geographical pairing was observed only for S.2649 and S.2650 from Illubabor. Cluster two composed Tafarikela, the material considered as standard for the study with S.2601, S.2600, S.2602, S.2642, S.2644 and S.2608. Geographical groupings were noted only for S.2600 and S.2601 from Harar province sub-clustering with Tafarikela in the cluster two. No duplications were noted among the germplasm studied.

[IV] DISCUSSION

Extent of distribution, areas of sampling and plant characteristics, breeding behavior and generation time are some of the important parameters that determine the level of genetic variability in a species. Because *C. arabica* is an allotetraploid and predominantly self-pollinated species, a high degree of genetic uniformity is expected [3, 14]. Molecular markers revealed the loss of genetic diversity due to the history of *C. arabica* cultivation, the self-pollination and selection by farmers [15, 16].

In coffee, there have been no alternatives to *ex situ* field collections for long-term germplasm conservation, due to recalcitrant seeds. If conventional methods of seed storage are used, the *C. arabica* seeds are known to be viable for a maximum of two to three years [17]. In *ex situ* field collections

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there is a risk of losing valuable germplasm due to diseases, pests and natural disasters as well as to poor adaptation to the local environment. Dulloo *et al.* [18]. have described strategies for *in situ* conservation of *Coffea* species, the *in situ* approach of plant genetic conservation has been recently emphasized [19]. *Ex situ* and *in situ* conservation strategies are complementary and should not be viewed as antagonistic [20]. The *in situ* method allows continuing evolution of the species in its natural habitat in order to allow perpetuation and integration of co-adapted gene complexes [19]. The *ex situ* approach, on the other hand, safeguards the species genetic diversity in case of possible habitat destruction and represent a readily available source of germplasm for research and breeding.

Table: 3. Shannon diversity indices for RAPD markers studied in the Ethiopian coffee germplasm

CCRI accession number	Province details	Taxa (S)	Shannon diversity index (H')
S.2440	Abyssinian province	66	4.19
S.2441		64	4.15
S.2600	Harar province	69	4.23
S.2601		72	4.27
S.2602	Shoa province	67	4.20
S.2615		73	4.29
S.2604	Sidamo province	57	4.04
S.2608		81	4.39
S.2642	Kaffa province	60	4.09
S.2644		71	4.26
S.2649	Illubabor province	79	4.36
S.2650		74	4.30
S.2707	Gojjam province	69	4.23
S.2709		69	4.23
S.2708	Eritrea province	80	4.38
Agaro*	----	76	4.33
Cioccie*	----	77	4.34
Tafarikela*	----	56	4.02
Mean±sd	----	70±7.4	4.23±0.10

* Comparative standard; Standard deviation (sd)

In order to quantify the level of polymorphism between wild coffee germplasm, the Dice coefficient of similarity was used to generate a similarity matrix **[Table–2]**. The genetic distances at the accession level fell in the range of 0.63 to 0.95 indicating a high level of polymorphism among the accessions. Lashermes *et al.* [14] reported a comparable level of molecular polymorphism in wild accessions of *C. arabica*. Anthony *et al.* [3] also reported a similar level of polymorphism among 80 accessions of *C. arabica* derived from spontaneous and sub-spontaneous trees in Ethiopia. In the present study Shannon diversity indices **[Table–3]** were in the range of 4.02 to 4.3, suggesting a relatively higher level of diversity in the wild populations, this holds good since the materials used in the study are collected in the wild without any bias towards the agro morphological characters. Present study has demonstrated that the RAPD technique could be applied for measuring the degree of variability in the wild coffee genetic resources from Ethiopia conserved at CCRI gene bank, India. The RAPD profiles generated in the present study showed moderate to high levels of DNA polymorphism which are in agreement with the morphological studies as per the IPGRI format by Dinesh *et al.*, [1–.2] Thus, for further improvement of coffee in India, we suggest the maintenance of Ethiopian germplasm showing good genetic variability in the gene bank.

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In the present study, relatively large differences were observed among the germplasm accessions collected in the Harar and Kaffa provinces (south west highlands of Ethiopia) in comparison to the other accessions from Illubabor, Gojjam and Eritrea and no duplications were noticed from the different collection localities of Ethiopia. Similar observations have been reported from agro morphological data [1, 22].

Since coffee plant is not indigenous to India any further improvement of existing coffee varieties or development of new varieties follows the path of hybridization with the plant of desired character. Results of the present study and earlier studies on morphological parameters [1, 21] are of potential importance in developing a more comprehensive breeding programme for *C. arabica* and Gene Bank maintenance in India as molecular profiles and morphological character profiles become parallel and collateral. Interspecific hybrids can be used as bridge genotypes to transfer desired traits from wild germplasm [1, 21] to cultivated forms and can further augment the crop improvement programme in coffee.

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